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Mechanisms of immune stimulation by bacterial DNA

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Introduction

DNA is a complex macromolecule whose immunological properties result from sequence microheterogeneity. Although mammalian DNA is immunologically inert, DNA from bacterial sources displays powerful immunostimulatory activities that rival those of endotoxin in their range and potency. These activities result from short sequence motifs, termed CpG motifs or immunostimulatory sequences (ISSs), that occur much more commonly in bacterial than mammalian DNA. Since these sequences can activate the innate immune system, bacterial DNA may serve as a "danger signal" to stimulate host defense during infection [25].

The activities of bacterial DNA have attracted great interest among immunologists because of their implications for host defense as well as the burgeoning use of DNA to prevent and treat disease. DNA forms the basis of a variety of novel therapeutic approaches, including antisense therapy, DNA vaccination, DNA adjuvants as well as tumor immunotherapy. Some of these approaches are already in clinical trial, with the development of DNA vaccines and DNA adjuvants likely to accelerate in the coming years.

Despite the rapid introduction of DNA into the clinical arena, the mode of action of immunostimulatory DNA is still not well understood. Many questions remain about the structure-function relationship of ISSs, the mechanisms of cell uptake and signaling mechanisms operative in different cell types. Furthermore, although human use of immunostimulatory DNA is clearly in the offing, much of the basic biology of this system has involved animal models. Whether conclusions drawn from animal studies will apply to man will require much further investigation.

To provide a perspective on the role of DNA in host defense and immunotherapy, this chapter will review information on the mechanisms of cell activation by immunostimulatory DNA, focusing on three main issues: (1) cellular targets of immunostimulatory DNA; (2) structure-function relationships of immunostimulatory DNA; and (3) signal transduction mechanisms. As this account will indicate, immune properties of DNA result from short sequence motifs that can directly or indirectly trigger B cells, T cells, NK cells, macrophages and dendritic cells. The consequence of these activities is the stimulation of host defense mechanisms important in the eradication of infection and possibly malignancy.

Cellular targets of immunostimulatory DNA

The original observations establishing the immunostimulatory properties of bacterial DNA came from experiments to define the anti-tumor activity of an extract of *Mycobacterium bovis* BCG. This extract, called MY-1, could promote the in vivo rejection of transplantable tumors in mice. As shown by biochemical fractionation, this extract was predominantly DNA. Furthermore, digestion of the extract by DNase could eliminate activity, suggesting that mycobacterial DNA itself could cause tumor rejection. Since the extract did not directly affect tumor cell growth, an action on the immune system provided the most likely explanation for the observed effects in vivo [27, 28, 35].

Subsequent studies demonstrated that the MY-1 extract augmented NK cell activity, although this activation was secondary to cytokine production by an adherent cell population. As shown by assays of cytokines as well as the effects of anti-cytokine antibodies, IFN-α/β was a major factor leading to NK cell activation. Since the induction of IFN-α/β and NK cell activity was a property of DNA from bacterial, but not mammalian sources, these seminal experiments clearly showed that bacterial DNA can exert immunostimulatory properties that do not simply reflect its polyanionic nature [42].

To delineate the structural basis of immunostimulatory DNA, the in vitro activity of sequences from cloned mycobacterial DNA was tested. These studies demonstrated that stimulation of NK cell activity results from a variety of sequence motifs that in general center on a CpG motif and have features of a palindrome. Importantly, these motifs are active in the form of short oligonucleotides (30 bases or less) and in vitro can mimic the effects of intact bacterial DNA in terms of stimulation of IFN production as well as NK cell activation [18, 36].

As shown in in vitro experiments using murine cell preparations, the activity of bacterial DNA extends to B cells. Thus, DNA from a variety of bacterial DNA species can induce in vitro mitogenesis and polyclonal B cell activation. This activity is sensitive to DNase but resistant to polymixin B. Furthermore, the mitogenic activity is T cell independent since elimination of T cells by anti-T cell reagents is without effect. Since cells from endotoxin-resistant C3H/HeJ mice show similar responses as those of other strains, these findings suggest that bacterial DNA can directly activate B cells by a pathway distinct from that of LPS [21].

Subsequent studies on B cell mitogenesis explored the sequence requirements for B cell activation using synthetic oligonucleotides as in vitro stimulants. By searches for common features of active compounds, Krieg and colleagues demonstrated that a 6-base motif centered on a CpG dinucleotide is immunostimulatory and can elicit both polyclonal B cell activation and antibody production. This sequence has the general structure of two 5' purines, an unmethylated CpG dinucleotide, and two 3' pyrimidines (Pu-Pu-CpG-Pyr-Pyr) and closely resembles the sequence responsible for IFN induction [16, 22].

The active sequence, defined either as a palindrome or CpG motif, provides an explanation for how bacterial DNA can be recognized as foreign and stimulate immunity. Thus, bacterial DNA and mammalian DNA differ markedly in their content of CpG motifs. At least two reasons account for the differences in the content of CpG motifs in eukaryotic and prokaryotic DNA. In mammalian DNA, cytosine and guanosine occur in tandem much less commonly than predicted by the base composition of DNA. This phenomenon is known as CpG suppression. Furthermore, in mam-

malian DNA, cytosine is commonly methylated in this position [10]. The result of these two features leads to a major quantitative difference in the presence of immunostimulatory sequences in eukaryotic and prokaryotic DNA.

While the basis of CpG suppression is not known, cytosine methylation has been considered an important regulatory element in determining the transcriptional activity of genes during differentiated cells function. The delineation of immune potential of CpG DNA, however, has suggested that CpG suppression and cytosine methylation may have evolved in concert as an immunological recognition system to allow bacterial DNA to signal the presence of infection [6]. This function of this alternative genetic code is speculative at present, but this line of thought has given impetus to elucidate mechanisms of immune activation by DNA.

Although the exact sequences of ISSs remain a matter of inquiry (see below), these findings have nevertheless suggested that short, synthetic oligonucleotides (oligos) can be used to elucidate immune activation by bacterial DNA. These synthetic compounds facilitate analysis of structure-function relationships and eliminate potentially confounding factors from contamination by material such as endotoxin. In the use of synthetic oligos, both phosphodiester (Po) and phosphorothioate (Ps) compounds have been used. Phosphorothioate compounds are DNA derivatives in which one of the non-bridging oxygens in the phosphodiester backbone is replaced by a sulfur atom [31]. These compounds are resistant to nuclease and may have a greater potency both in vitro and in vivo than phosphodiesters.

Analysis of immune activation by DNA has variously used natural DNA, Po oligos and Ps oligs. Although these DNA sources may not be identical in either mode of action or structure-function relationships, they exhibit sufficient similarity to allow results obtained with these different stimulants to be interpreted together. A subsequent section will consider differences among these compounds. For convenience, these DNA molecules will be called CpG DNA.

Cellular effects of CpG DNA

CpG DNA exerts direct or indirect effects on all major cellular elements of the immune system, leading to enhanced activity of B, T and NK cells as well as macrophages and dendritic cells as described below.

Macrophages

Similar to endotoxin, CpG DNA powerfully activates macrophages and induces cytokines whose downstream actions influence other cellular responses. The cytokines stimulated by CpG DNA include IFN-α/β, IL-1β, IL-6, TNF-α and IL-12. In addition to the production of cytokines, macrophages stimulated by CpG show enhanced expression of inducible nitric oxide synthase (iNOS) as measured by both mRNA as well as protein production. In the induction of iNOS by CpG DNA, IFN-γ may be required at least in certain cell lines used as models for this response [8, 30, 33].

Although stimulation of macrophages by CpG DNA and endotoxin leads to a similar array of inflammatory mediators, these responses are distinct. Evidence for this conclusion comes from the ability of endotoxin-resistant C3H/HeJ mice to re-

spond to DNA. Furthermore, among macrophage cell lines, responses to CpG DNA and endotoxin are not invariably linked. The differences in activation by CpG DNA and endotoxin are also apparent in the pattern of IL-12 p40 transcription [5].

B cells

CpG DNA is mitogenic for murine B cells and can induce polyclonal immunoglobulin production. These responses are independent of T cells as shown by the responses of T-depleted cultures and B cell lines. Immunoglobulin production by B cells may result from the effects of IL-6 which is also induced by CpG DNA. This cytokine may function in an autocrine fashion since anti-IL-6 can eliminate production of Ig, although this treatment does not affect proliferation [16, 21, 44].

The effects of CpG extend to the process of apoptosis. In the WEHI-231 B cell line, CpG oligos protect against apoptosis resulting from stimulation by anti-IgM. In primary B cells, CpG DNA can protect CD40 ligand-stimulated B cells from Fas mediated cytotoxicity induced by either cytotoxic T cells or anti-Fas antibodies. This protection may result from down-regulation of Fas as demonstrated by FACS measurement of cell surface Fas expression. Although CpG DNA up-regulates IL-6, the anti-apoptotic effects are independent of this cytokine since neither IL-6 nor supernatants of CpG DNA-stimulated cells reproduce the effects of the DNA [37, 45].

NK cells

Since purified NK cells do not respond directly to CpG DNA, induction of cytotoxicity and IFN-γ production by these cells appears to be the consequence of downstream effects of cytokines induced by this stimulant. Among these cytokines, IFN-α/β, IL-12, TNF-α, all inducible by CpG DNA, can activate NK cells to cytotoxicity and IFN-γ production. Similar to findings with other cell types, CpG DNA may interact with other stimulants (e.g., cytokines) to promote cellular responses. Thus, NK cells in the presence of IL-12 may have an augmented response to CpG DNA [1, 4, 40].

T cells

T cells alone do not appear to respond directly to CpG DNA, although their activity can be influenced by cytokines produced by other cell types (e.g., macrophages) activated by CpG DNA. In addition, CpG DNA may co-stimulate T cells that have been activated through their T cell receptor. In in vitro systems in which antigen-presenting cells (APCs) are eliminated, CpG DNA can induce IL-2 production, IL-2 receptor expression and proliferation of purified T cells stimulated by anti-CD3. This stimulation occurs in both CD4 and CD8 cells and can be inhibited by cyclosporine A. Since T cells from CD28 knockout mice show proliferation by CpG DNA and anti-CD3, CpG may be able to substitute for CD28 co-stimulation [2].

Dendritic cells

Like macrophages, dendritic cells appear to respond directly to CpG DNA. As shown using dendritic cells derived from bone marrow cultures, CpG DNA can stim-

ulate both immature and mature populations as determined by flow cytometry. For both populations, CpG DNA can induce up-regulation of MHC class II, CD40, and CD86 cell surface molecules, although expression of CD80 is not affected. Furthermore, CpG DNA can stimulate both mature and immature populations to produce IL-12, IL-6 and TNF-α. As a consequence of these effects, dendritic cells activated by CpG DNA demonstrate enhanced APC function in mixed lymphocytes reactions as well as induction of T cell responses by staphylococcal enterotoxin B [29].

Together, these observations indicate that CpG DNA can promote immune responses of the Th1 kind through direct effects on APCs and B cells as well as indirect effects on other cytokines, most notably IFN-γ. Since these effects are central to the use of CpG DNA either alone or as part of a DNA vaccine, elucidating these mechanisms has become a major investigative priority. Two important questions must be addressed to refine the use of CpG DNA: (1) What are the structures of DNA key to immunomodulation? and (2) What are the intracellular mechanisms for immune activation? The subsequent sections will review these topics.

Structure-function relationships of immunostimulatory DNA

The sequences of ISSs have been investigated intensively since defining their structure-function relationship is an important prelude to characterizing any receptors or binding molecules that interact with CpG and lead to activation. In this endeavor, synthetic oligonucleotides have become a mainstay. At this point, hundreds if not thousands of different oligos have been tested in vitro or in vivo. While this mass of information should provide clarity on this issue, important questions remain concerning the structure of motifs conferring activity.

At least two groups have extensively investigated structure-function relationships of CpG DNA. Tokunaga, Yamamoto and colleagues originally tested oligonucleotides bearing sequences from mycobacterial genes and determined common features associated with activity. These investigators then systematically varied these sequences in the form of 30-mer oligonucleotides in which flanking sequences were held invariant. Using NK cell activity as an assay, palindromic sequences showed the greatest potency in vitro. In general, these sequences had the Pur-Pur-CpG-Pyr-Pyr structure, although there were exceptions to this rule. Thus, the sequences CGATCG and ATCGAT both showed activity [12, 18, 41].

In contrast to this approach, Krieg and colleagues derived rules for activity by inspection of a large panel of oligonucleotides tested for activity primarily using B cell proliferation assays [16]. On the basis of these data, these investigators identified the Pur-Pur-CpG-Pyr-Pyr motif as the most critical for activity. While this motif accommodates many palindromes, a palindromic sequence was not found to be essential for activity. In studies by both groups, methylation of the cytosine residue was associated with loss of activity. Furthermore, at least some sequences identified on the basis of murine studies were also active in humans, using cytokine production and NK cell activation as readouts. Human B cells, however, do not seem to respond to either bacterial DNA or Po oligos [19].

Since compounds designed on the basis of either motif produce useful immunostimulants, the difference in interpretation of this issue relates to understanding mechanisms of stimulation. The studies by Tokunaga, Yamamoto and colleagues suggest that a higher order base-paired structure may be the stimulatory moiety. In

contrast, the studies of Krieg and colleagues point to a linear sequence that may or may not have the capacity for base pairing.

Several aspects of this controversy bear comment. First, the focus on the 6-base motif minimizes the contribution of the flanking regions. These flanking regions may affect the conformation of the 6-base motif or exert other effects on the motif's activity. Comparing oligos that differ in length as well as flanking sequence may, therefore, lead to confusion about the intrinsic activity of the 6-base motif. Furthermore, while it is generally assumed that the structure-function relationships for stimulation of B cells are the same as for macrophages, this point has not been rigorously evaluated. Indeed, there is evidence that certain sequences can stimulate IL-12 but not TNF-α [20]. Some of the differences in structure-function relationships of compounds may, therefore, relate to the cell system used for assay.

Another issue in evaluating the structure of ISSs concerns the use of Ps oligos as test compounds. These compounds, which have the substitution of a sulfur for one of the non-bridging oxygens in the DNA backbone, differ from Po compounds in properties such as nuclease resistance, melting temperature, protein binding and cellular uptake. Available data suggests that rules for activity of Po and Ps oligos differ, since some Ps and Po oligos of the same sequence vary in their capacity to stimulate cells. Furthermore, even a limited stretch of Ps sequence in a Po oligos may alter the activity of an ISS and in some instances inhibit responses [1, 9].

In view of structural and pharmacological differences of Po and Ps compounds, any assessment of structure-function relationship must consider the impact of backbone chemistry. B cells responses provide a telling example of this point. Thus, in mouse, stimulation of murine B cells by Ps oligos is not directly related to the content of CpG motifs and can occur in their absence [3, 23]. This finding suggests primacy of the backbone in certain systems. Similarly, in humans, Ps compounds are mitogenic for highly purified B cells, whereas Po compounds are inactive; optimal activity also does not require a CpG motif for these cells [19].

Adding to the complexity of this system, recent data indicate the existence of DNA sequences that can neutralize or inhibit the immunostimulatory activity of CpG motifs. These sequences were discovered in studies on the immune activity of adenoviral DNA, which showed that, while type 12 adenoviral DNA is active, type 2 and type 5 DNA fails to stimulate responses of human peripheral blood. Since type 2 and type 5 DNA both contain CpG dinucleotides, their lack of activity suggested the presence of other DNA sequences that could neutralize or block stimulation. The ability of the non-stimulatory adenoviral DNA to block the response to *Escherichia coli* DNA supported that possibility. As shown by sequence comparisons of adenoviral DNA and as well as the effect of synthetic oligos, neutralizing sequences may result from direct repeats of CpG dinucleotides as well as CpG motifs preceded by a C or followed by a G [15]. While suggesting a strategy for viruses to subvert host defense, these motifs are also relevant in the design of DNA vectors for vaccination or gene therapy.

These considerations suggest that immune stimulation by DNA is the summation of stimulatory and inhibitory activities that is not solely related to the presence of CpG motifs. For example, unmethylated mammalian DNA fails to stimulate cells despite its content of CpG motifs, possibly because neutralizing or inhibitory sequences prevent activity [32]. Similarly, among bacterial DNA, potency of immune stimulation varies markedly depending on species, with some DNA having only limited ability to induce mitogenesis or cytokine production despite the presence of CpG

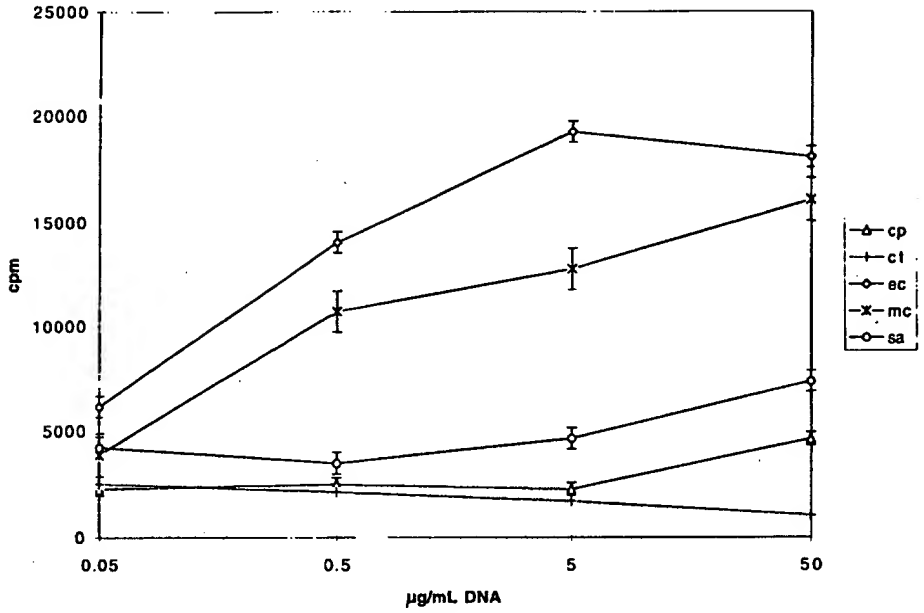

Fig. 1. Stimulation of in vitro proliferation by bacterial DNA. In vitro mitogenic activity of bacterial DNA was assessed by proliferation assays of murine spleen cells. DNA tested included *Clostridium perfringens* (*CP*), calf thymus (*CT*), *E. coli* (*EC*), *Micrococcus lysodeikticus* (*MC*) and *Staphylococcus aureus* (*SA*). Reproduced with permission from [24]

motifs [24]. Figure 1 illustrates this point. Until structure-function relationships of immunostimulatory DNA are better defined, these observations suggest caution in equating CpG content with stimulation and assuming that bacterial DNA are uniform in activating potential.

Influence of base sequence on cell binding and DNA uptake

Several lines of evidence suggest that immune activation by CpG DNA requires DNA internalization. This evidence includes the following observations; (1) DNA attached to beads fails to stimulate cells; (2) agents such as lipofectin that can promote cellular uptake of DNA augment immunostimulation; (3) cell binding of CpG and non-CpG DNA is the same; and (4) the ability of CpG DNA to stimulate different immune cell populations may be related to extent of cell binding and of endocytosis [14, 43]. Thus, resting T cells, which are unresponsive to oligos unless they receive another activating signal, are nonendocytic and show lower cell surface binding of oligos than B cells.

While the uptake and trafficking of DNA are not well understood, receptor-mediated endocytosis appears to be the predominant mechanism by which DNA enters cells. In general, this process has been considered to be sequence nonspecific and, therefore, not a factor influencing immune activation by DNA. Studies on both synthetic and natural DNA indicate, however, that certain DNA sequences can promote uptake of DNA and can significantly influence the activity of CpG DNA.

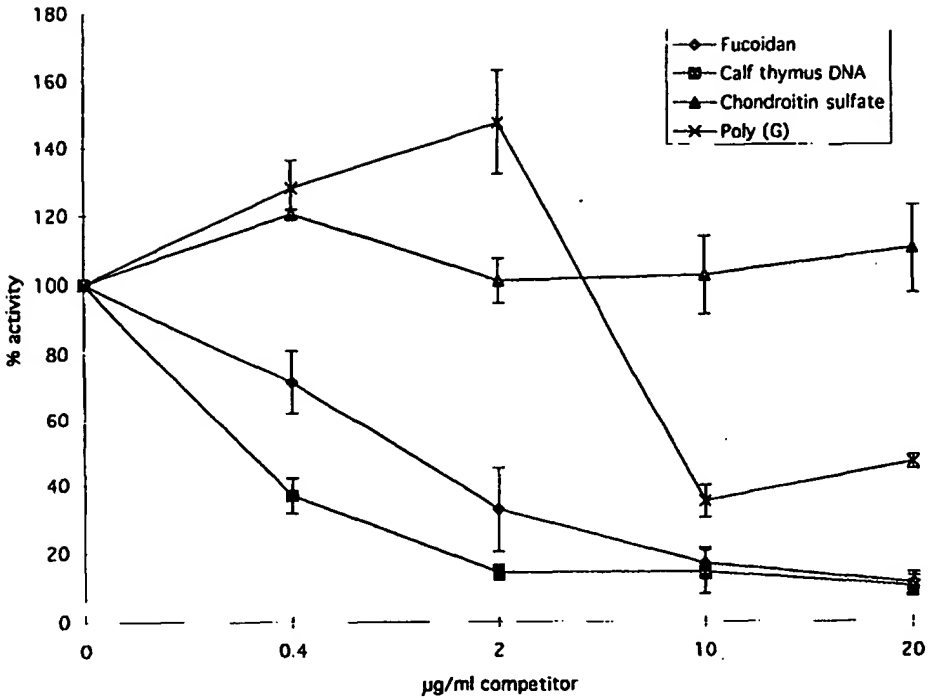

Fig. 2. Effects of MSR ligands on in vitro IL-12 responses induced by plasmid DNA. The effects of MSR ligands on IL-12 production were assessed in cultures of murine spleen cells stimulated with pCMV-β plasmid. Results are reported in terms of % activity of uninhibited cultures. Reproduced with permission from [39]

Furthermore, certain cell surface receptors may have enhanced binding to DNA on the basis of sequence, pointing to another factor determining the structure-function relationship to CpG DNA.

The clearest evidence for the impact of sequence on binding comes from observations on the effects of flanking bases on the activity of the palindromic sequence AACGTT embedded in 30-mer oligos in which the flanks consisted of single bases. In oligos of this structure, dG flanks led to the highest activity when assessed using NK cell activation as a measure. Other flanks (e.g., dT) caused appreciable activity, although dA flanks were inactive. Since extended runs of each base were inactive, these findings suggest that flanks can modify the activity of a CpG motif without themselves displaying causing immunostimulation [13].

While a variety of mechanisms could account for the ability of dG runs to enhance immunostimulation by a CpG motif, enhanced cell binding and uptake appears most likely. Extended runs of dG, by virtue of their ability to base pair with each other, can form four strand arrays called G-quartets or quadruplex DNA. In these arrays, strands can align in either the parallel or anti-parallel orientation. Such a structure may exist at telomeres where the concentration of dG runs is high [38].

Enhanced binding of dG runs to cells may relate to binding to both surface receptors as well as the phospholipid bilayer. Among macrophage cell surface molecules, the type A macrophage scavenger receptor (MSR) displays a broad pattern of polyan-

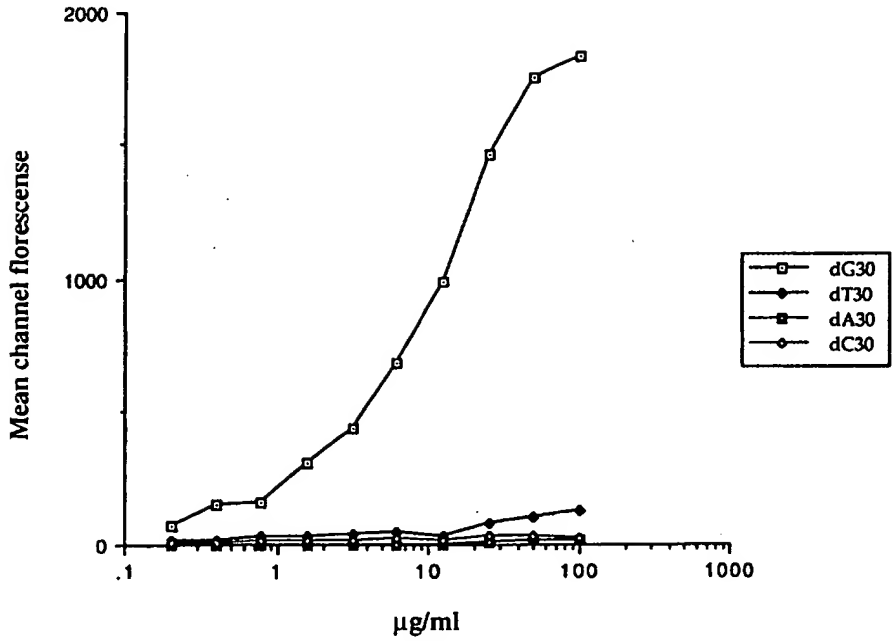

Fig. 3. Flow cytometry analysis of oligonucleotide binding to murine spleen cells. The binding of fluoresceinated single-base 30-mer oligonucleotides to murine spleen cells was assessed. Results are reported in terms of mean channel fluorescence. Reproduced with permission from [26]

ion binding and has specificity for oxidized low-density lipoprotein (LDL), acetylated LDL, dextran sulfate, fucoidan, LPS and poly I and poly G. This receptor may serve as a pattern recognition molecule and take up either foreign or damaged self proteins for elimination. Although LPS binds the MSR, it does not cause cell activation suggesting the role of the MSR as a clearance rather than triggering molecule [17].

In in vitro culture, the MSR appears to play a role in the response to CpG DNA. Thus, short oligos with dG residues show enhanced binding to macrophages and can cause activation by a mechanism that is blocked by other MSR ligands. Furthermore, although the MSR has strong preference for dG runs, it can also bind natural DNA [34, 39]. Thus, similar to effects of oligos, MSR ligands can block the response of macrophages to bacterial as well as plasmid DNA (Fig. 2). At present, it appears that the role of the MSR is to promote uptake of DNA into cells rather than signal activation since MSR ligands other than CpG DNA did not cause cytokine production.

The effects of dG residues also extends to B cells, although these cells lack the type A MSR. In contrast to results with macrophages, dG oligonucleotides, even in the absence of CpG motifs, can cause B cell proliferation and antibody production under conditions in which other single-base oligos are inactive [22]. Furthermore, among 30-base compounds with CpG motifs, dG flanks cause the highest level of cell activation. The extent of activity is correlated with cell surface binding. As shown by flow cytometry (Fig. 3), dG oligos (with or without a CpG motif) show the highest level of binding [26].

Studies with synthetic phosphorothioates indicate that dG oligos have greater interaction with phospholipid micelles than other single-base oligos. This finding

suggests that increased cell binding of dG runs could reflect either binding to receptors or the lipid bilayer [11]. While indicating clearly that base composition can influence the magnitude of cell binding of DNA, these findings also demonstrate differences in the activation requirements for macrophages and B cells and the ability of non-CpG sequences to trigger activation in at least certain cell types.

Signal transduction pathways

As this account indicates, DNA signals a cell in a sequence-(or structure-) specific manner following internalization. This internalization involves maturation of an endosomal compartment, since inhibitors of endosomal acidification such as chloroquine and related compounds can block the ability of CpG DNA to induce secretion of IL-6 or prevent apoptosis induced by anti-IgM. Furthermore, while activation of IL-6 does not involve protein kinase C, protein kinase A or nitric oxide, it is associated with the generation of reactive oxygen species (ROS). The generation of ROS, which can also be blocked by chloroquine, appears to be an important step in transcriptional activation since antioxidants such as PDTC are also inhibitory [46, 49].

As shown in cell lines as well as bone marrow macrophages, CpG DNA can increase NK-κB binding activity as shown by EMSA as well as the transcriptional activity of luciferase reporter genes under the control of the HIV-1 LTR. Similarly, in J774 cells, CpG DNA can induce NF-κB as well as prevent the degradation of IκBα and IκBβ. The importance of NF-κB in activation is further demonstrated by the effects of various inhibitors of steps in NF-κB activation. PDTC, an inhibitor of IκB phosphorylation;TPCK, a protease inhibitor of IκB degradation; and gliotoxin, an inhibitor of IκB degradation can all block the induction of cytokines such as IL-6. Since anti-oxidants block the activation of NF-κB induced by CpG DNA, these findings suggest that ROS generation precedes NF-κB activation and is required for its expression [30, 46, 47, 49]. The mechanisms for ROS generation cannot be delineated from these experiments.

While NF-κB activation could explain some of the observed activation by CpG DNA, other pathways appear to be critically involved in this process. In murine macrophages and dendritic cells, CpG DNA can induce the phosphorylation of Jun N-terminal kinase (JNK) kinase 1 and subsequent activation of stress kinases JNK 1/2 and p38. Furthermore, as shown using reporter gene constructs, the activation of stress kinases is associated with an increase in AP-1 transcription activity and the phosphorylation of c-Jun. Since inhibitors of p38 kinase activity can inhibit the expression of TNF-α and IL-12, this pathway must also contribute to transcriptional activity by CpG ODN. As in the case of NK-κB, the stimulation of stress kinases is dependent on the endosomal maturation and can be inhibited by chloroquine and bafilomycin [7, 48].

While NF-κB and stress kinase pathways account for transcriptional activation by CpG motifs, they do not yet explain the sequence specificity of triggering. The activity of DNA of only certain structures (sequence or palindrome) has suggested the existence of a binding protein(s) that could serve as proximal element in signal transduction, coupling internalization of DNA with activation of NF-κB or stress kinases. With identification of such binding and coupling molecules, the activation pathways will greatly clarify and allow the development of more potent activators or inhibitors of this system.

Conclusion

Bacterial DNA promotes powerful immune system activation because of its content of characteristic sequence motifs. These sequences induce cellular changes that promote inflammation and the generation of Th1 responses. This activation involves internalization of DNA as well as the activation of NF-κB and stress kinases. Future studies will identify DNA-binding molecules that are key to these activation events and may serve as targets for novel immunomodulatory agents.

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